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The growth of cells in the body is closely regulated by peptide growth factors, which are detected by the cells via cell-surface receptors. Many human cancers of the breast, brain, etc., can develop if these receptors behave as if they sense the presence of growth factors when they should not. One particular family of receptors that has been heavily implicated in the development of human cancers is the erbB receptor tyrosine kinase family, which includes both the EGF-Receptor and erbB2/HER-2/neu - both of which are major targets for chemotherapeutic agents either in clinical use or in clinical trials. The physiologic outcome of both normal and oncogenic erbB receptor activation depends on the ability of these receptors to form both homo- and heteromeric complexes, but the exact mechanism underlying the formation of these complexes is not well understood. We are attempting to use a variety of cell biological, biochemical and biophysical approaches to elucidate the nature of homo- versus heteromeric complexes in the erbB receptor family.

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INTRODUCTION

The erbB family of receptor tyrosine kinases has been shown to be clinically important in all stages of life, from development of the embryo and fetus to the maintenance of normal adult physiology (1, 2). However, these receptors have also been implicated in a vast array of human cancers, affecting a wide diversity of tissues and organs, including breast, prostate, brain and lung (3). The role of erbB2 (also known as HER-2 or neu) has been extensively studied in breast cancer, leading to the development of Trastuzumab (Herceptin), an FDA approved humanized monoclonal anti-erbB2 antibody, for use in chemotherapy against erbB2-expressing breast cancer (4). However, this drug has been proven to have only limited clinical efficacy, thus underscoring the need for further research into the mechanisms of erbB receptor signal transduction. In this project we are attempting to use a wide variety of techniques from cell biology, biochemistry and biophysics in order to gain a better understanding of the mechanisms underlying the immediate-early steps in erbB receptor activation, namely the formation of homo- and heteromeric complexes. It is hoped that by elucidating more detailed information about these interactions at the structural and molecular level, we will be able to identify specific targets for drug discovery which will ultimately lead to improved chemotherapeutics for use in erbB receptor-expressing cancers.

BODY

The aim of this project is to determine differences, if any, between homo- and heteromeric interactions of the erbB receptor tyrosine kinases. The original experiments, as described in last year's annual summary, were performed in a panel of five different breast cancer cell-lines. However, we experienced great difficulty interpreting data from these cell-lines given the inherent differences from one cell-line to another, genomic instability of cancer cells, interactions between endogenously expressed receptors and the exogenous receptor constructs we wished to study, and a lack of reproducibility of results using these cell-lines. As such, we have moved to using a novel cellular background for our studies. Our current work focuses on identifying components of receptor oligomerization in the Drosophila Schneider 2 (S2) cell-line. S2 cells offer a variety of advantages for studying erbB receptor oligomerization over human cancer cells. These cells express no detectable levels of erbB receptors and are not responsive to human erbB receptor growth factors. The cells are genetically quite stable and are easily manipulated. Finally, the S2 cell-line provides us with a uniform background in which to complete all of our experiments, so the results from different experiments can be directly compared to one another.

As we proposed in the Statement of Work, we are interested in examining differences in the receptor complexes formed upon stimulation with epidermal growth factor (EGF) and neuregulin 1-β1 (NRG). We have been unable to identify any cellular responses to these growth factors by the S2 cells, so we are confident that any responses we may detect will only be due to exogenous human erbB receptor expression. We would like to use these cells to specifically address the erbB2-erbB3 heterodimer, which is well documented to be the preferred heterodimeric complex involving erbB2 and which has also been described as the pair with the highest mitogenic potential (5- 8). The erbB2-erbB3 pair has also been marked as especially important in the development breast cancer, given that recent studies indicate that a significant percentage of breast cancers overexpress both erbB2 and erbB3 (9).

We are interested to examine the nature of the erbB2-erbB3 interaction. Recent crystal structures of the extracellular portions of these receptors demonstrate that erbB2 exists in a conformation that appears to be poised for dimerization, even in the absence of growth factors (10, 11). This is consistent with the fact that erbB2 has no known ligand, yet is able to form homo- and heteromeric interactions with other erbB receptors on the cell surface. The crystal structure of erbB3, on the other hand, showed that erbB3 exists in a "tethered" conformation in the absence of growth factor (12). Structural data from various labs on the EGF-Receptor (EGFR, or erbB1) extracellular domain indicates that these receptors remain largely in this "tethered" conformation until they bind growth factors, at which time the extracellular domains open into an "extended" conformation which reveals a "dimerization arm" capable of mediating interactions with other molecules (13-15). It is therefore believed that erbB3 will also open into an extended conformation upon binding NRG, allowing it to form oligomers. It is the precise nature of these oligomers that we are interested in.

Biophysical studies of recombinant erbB receptor extracellular domains have been unsuccessful in demonstrating that erbB3 forms homodimers (16, 17). However, cellular studies have, in an indirect fashion, hinted that formation of these erbB3

homodimers does actually take place on the cell surface (18). This question is difficult to address, as the erbB3 cytoplasmic kinase domain is naturally inactive, so it is not possible to simply monitor the phosphorylation of erbB3 in cells in response to growth factors. We have chosen to bypass this impasse by making chimeric fusions of the erbB3 extracellular- and transmembrane-domains with the cytoplasmic domains (including the tyrosine kinase domain) of the EGFR (we call this fusion erbB3/erbB1). It has been well demonstrated that the EGFR tyrosine kinase domain is only activated in a dimeric complex, so NRG-stimulation of S2 cells expressing this chimera should only lead to phosphorylation events if the erbB3 receptor portions specifically dimerize in response to growth factor stimulation. As a positive control, we have also generated a similar chimera, fusing the extracellular- and transmembrane-domains of erbB4 to the cytoplasmic domains of the EGFR (erbB4/erbB1). ErbB4 is another NRG-binding member of the erbB receptor family that is well known to dimerize in response to growth factor stimulation.

The current status of this project is as follows: we have been able to successfully demonstrate that stimulation of S2 cells expressing full-length EGFR with EGF show auto-activation of the exogenous EGFR as well as activation of the Drosophila mitogen activated protein kinase (MAPK), a common downstream effector of growth factor receptors. These studies indicate that the S2 cell-line is an ideal cellular background for testing our chimerae as the receptors can apparently be expressed appropriately in the cell surface, become auto-activated upon growth factor stimulation, and that the human EGFR cytoplasmic domains can actually couple to downstream Drosophila signal transduction pathways. Expression of the different chimerae in the S2 cells provides us with interesting information – the erbB4/erbB1 chimera is capable of autoactivation and MAPK phosphorylation in response to NRG-stimulation, as predicted, whereas cells expressing the erbB3/erbB1 chimera remain unresponsive to NRG-stimulation (see Figure 1). This data indicates that erbB3 does not dimerize when it binds NRG, which provides resolution to an important question in the field.

Given that we now believe that erbB3 does indeed not form simple homodimers, we are interested in examining the nature of the erbB3-erbB2 heteromer. It has been previously shown that expression of erbB3 alone does not allow cells to respond to NRG, but co-expression of erbB3 and erbB2 creates a powerful receptor complex for the growth factor. We are currently in the process of co-expressing erbB2 in our cells that already stably express the erbB3/erbB1 chimera to monitor how these cells respond to NRG. This is important both as a control to establish that the erbB3/erbB1 chimera is a functional receptor, but also provides us with a useful tool for studying the actual interaction between these different receptors. In addition, it has been shown that if a form of erbB2 is instead expressed in which the kinase domain of erbB2 has been mutated to be catalytically inactive, neither receptor is phosphorylated in response to growth factor stimulation. We are therefore also in the process of co-expressing the kinase-inactive erbB2 receptor in the erbB3/erbB1 cells. These cell-lines will be used to address the interaction of the cytoplasmic domains of the receptors in order to gain a better understanding the origin of the different phosphorylation events.

As mentioned earlier, we have witnessed an explosion of structural data on the erbB receptor families in the last year. The structure of the "tethered," inactive form of the EGFR was solved by our lab (13). I was involved in mutagenesis studies that helped

us determine the actual mechanism by which binding of growth factor to the receptor extracellular domain actually leads to a conformational change allowing for the promotion of dimer formation.

I am currently pursuing an extension of this work to gain further understanding of the potential roles of different dimerization interfaces in the EGFR extracellular domain on ligand binding and dimerization. Figure 2 shows a model of the full-length EGFR extracellular domain in the ligand-bound, dimeric conformation. I have circled the four apparent dimerization interfaces. Site A is a glutamine at residue 194 (Gln194). Based on the structure of the dimeric conformation, this amino acid forms hydrogen bonds across the dimer to Gln194 of the other extracellular domain in the dimer. Site C is similar, but contains two residues – aspartic acid 279 (Asp279) and histidine 280 (His280). Site B is the "dimerization arm," which is believed to be the primary structure involved in dimerization (14, 15). Finally, site D is a putative dimerization loop identified by our lab's crystal structure. I am in the process of purifying recombinant soluble EGFR extracellular domains (s-erbB1) containing mutations at each of these sites. Mutations in the dimerization arm (site B) that inhibit dimerization and/or the ability of the EGFR to signal in cellular studies were described by the groups that solved the structures of the dimeric complex, but no studies of the roles of sites A, C or D have been described (14, 15). In addition, the studies of mutations at site B were qualitative, but did not provide any information as to the quantitative effects of these mutations. For sites A and C, we will mutate the indicated residues to alanine; site C mutants will be generated as described in the structure papers, and the putative dimerization loop at site D will be deleted. Our plan is to compare the ligand-binding ability and dimerization potential of these mutants with wild-type s-erbB1.

KEY RESEARCH ACCOMPLISHMENTS

- Generated Schneider 2 cell-lines stably expressing human EGFR, erbB2, erbB3, erbB4, erbB3/erbB1 chimera and erbB4/erbB1 chimera
- Performed MAPK and receptor activation experiments in stable S2 cell-lines, demonstrating lack of erbB3 homodimerization upon binding NRG
- Generated various mutants of soluble EGFR extracellular domain

REPORTABLE OUTCOMES

This research has resulted in the generation of a set of Drosophila cells expressing various functional human erbB receptors.

Work supported by this research resulted in the following publication:
Ferguson, K. M., Berger, M. B., Mendrola, J. M., Cho, H-S, Leahy, D. J., and Lemmon, M. A. (2003) *Molecular Cell*, **11**, 507-517.

CONCLUSIONS

The research completed to date has allowed us to draw some interesting conclusions about the nature of erbB receptor homo- and heteromeric interactions. The studies involving the erbB3/erbB1 chimera have already proven to demonstrate that the erbB3 receptor does not form homodimers in response to binding its cognate ligand, NRG. There has been some controversy in the field as to whether or not erbB3 does in fact form homodimers as there are conflicting reports between biophysical and cell biological experiments. This result will therefore serve to present a resolution to this conflict. In addition, it is clear that erbB3 is not only the preferred heterodimerization partner for the well-known oncogene erbB2, but the erbB3-erbB2 pair is the most mitogenic receptor complex for this family. In addition, recent data has shown that a significant proportion of human breast cancers overexpress both erbB2 and erbB3. It is therefore urgent that we gain a better appreciation for the mechanism of formation and activation of the erbB2-erbB3 heterodimer. Future studies with the cell-lines generated and those in the process of being generated, namely, cells co-expressing the erbB3/erbB1 chimera and either wild-type or kinase-inactivated erbB2 will allow us to further probe the nature of this interaction, and thereby identify targets for specific drug discovery.

The EGFR is already a well-described target for potential chemotherapeutic agents, as has been widely publicized in the current news media in regards to the ImClone anti-EGFR antibody Erbitux. In the past year a large number of crystal structures of erbB receptor members have been released, including two structures of the EGFR (erbB1) extracellular domain in its active, "extended," dimeric form, as well as a structure of the inactive, "tethered" conformation. Mutagenesis studies stemming from this project led to the deduction of the mechanism by which ligand binding leads to a stabilization of the extended form of the receptor, which is then poised in a conformation that allows for successful dimerization. Current studies of other mutations in the s-erbB1 molecule will allow us to gain a greater understanding of the roles of various potential dimerization interfaces in this molecule, which should hopefully highlight new regions of the molecule to specifically target in order to block dimerization, and therefore, activation, which is the key to blocking oncogenic signaling by this receptor.

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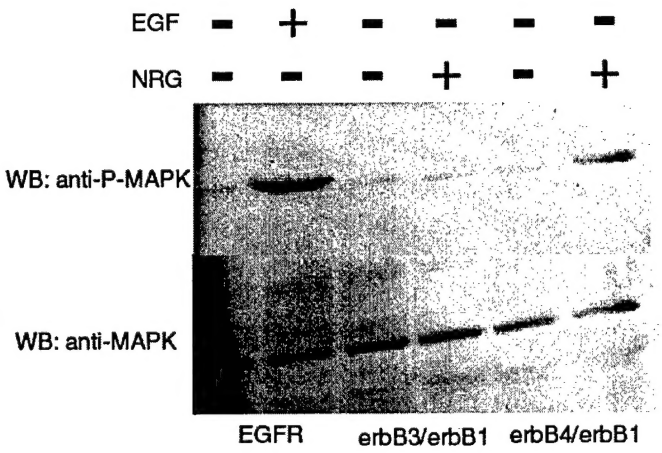
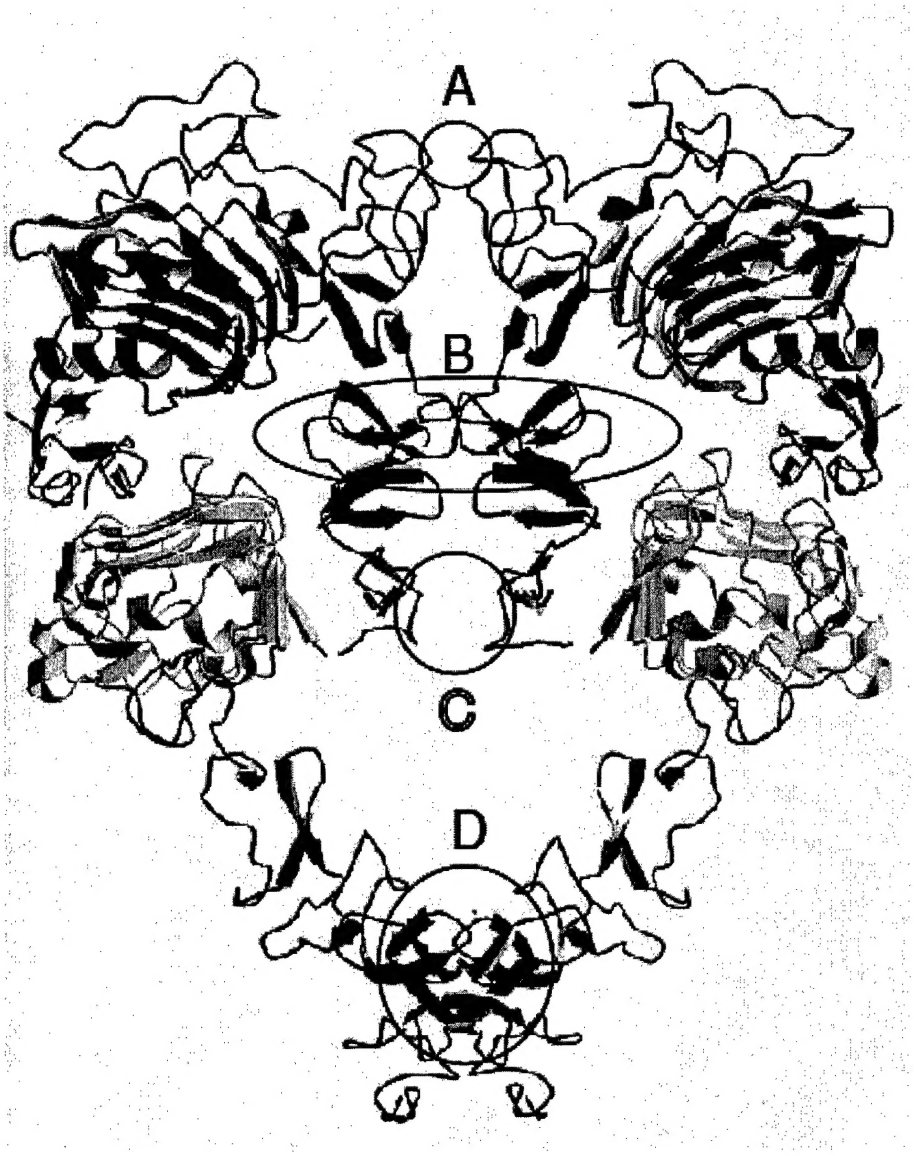
APPENDICES

FIGURE 1



Legend: Western Blot analysis of Drosophila MAPK phosphorylation by growth factor stimulation in S2 cells expressing exogenous human erbB receptor constructs. The top panel shows the levels of activated, phosphorylated MAPK in the cell lysates (P-MAPK) whereas the bottom panel shows the total amount of MAPK in the same lysates. The cell-lines are indicated below the figure and the growth factor stimulation-state is indicated above.

FIGURE 2



Legend: Model of the EGFR extracellular domain dimer. The circled regions (A – D) are dimerization interfaces discussed in the text.